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TITLE OF THE INVENTION (500 characters max)

MONOCLONAL ANTIBODIES FOR DETECTION OF URINARY TRYPSIN INHIBITORS

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Respectfully submitted, Date

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PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT

for

MONOCLONAL ANTIBODIES FOR DETECTION OF URINARY TRYPSIN INHIBITORS

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MONOCLONAL ANTIBODIES FOR DETECTION OF URINARY TRYPSIN INHIBITORS

Background of the Invention

This invention relates generally to the detection of urinary trypsin inhibitors (UTIs) in human urine. Urinary trypsin inhibitors are a group of Serine protease inhibitors found predominately in urine. This group of inhibitors primarily forms after an increase in the number of white blood cells in the body due to the release of elastase during infection or inflammation. UTIs are not normally found in the urine produced by healthy individuals. The amount is elevated in those whose bodies have bacterial infections and inflammatory disorders or other maladies such as malignant tumors, kidney disease, myocardial infarction, lung emphysema, surgical trauma, and kidney stones among others. Trypsin is a member of a family of Serine proteases, i.e. enzymes, that includes trypsin, elastase, kallikrein, plasmin, thrombin, chymotrypsin, and cathepsin, among others. UTIs inhibit one or more of the Serine proteases.

When infections and/or inflammation occur, the bodies' response involves the production of serine proteases such as elastase released by neutrophils. Non-inhibitor forms of UTI, called pro-inhibitors, such as interleukin-α-inhibitor (I-α-I) and the pre-interleukin-α-inhibitor (P-α-I), circulate freely in plasma of healthy and diseased individuals. Serine proteases cause proteolysis of the pro-inhibitors and release the lower molecular weight UTI into active function. The released urinary trypsin inhibitors act on serine proteases at the inflamed sites and are later excreted in the urine. Discovered in 1909, urine trypsin inhibitors are Kunitz type protease inhibitors and have been named HI-30, Mingin, Urinastatin, Serpin, and Ulinastatin over the years with the scientific community settling on the name Bikunin for a prevalent fragment of ~30 Kda molecular weight. The amino acid sequence of the Bikunin inhibitor fragment is known. It contains two Kunitz inhibitory binding domains and a large and variable chondroitin

sulfate chain. See the International Journal of Biochemistry and Cell Biology 32 (2000) 125-137.

Although measurement of the amount of urinary trypsin inhibitors has been done by several methods, e.g. enzyme inhibition, antibody stains, latex agglutination, and radioimmunoassays, all of the UTIs in the sample are measured. It has not been shown that certain forms of the inhibitor can result from chronic disease and are more easily distinguished from the inhibitors which were present in individuals with lesser degrees of inflammation. As a result the diagnostic use of UTIs has to date only been as a non-specific marker of infection and/or inflammation. The non-specific nature lessens the clinical utility as determination of UTIs does not help the care giver to know the type or site of infection and makes it difficult to separate conditions for which the patient would need therapy from general ailments. The present invention is directed toward making such distinctions.

One method of measuring the UTI content of a urine sample involves the addition to the sample of known amounts of trypsin and then measuring the degree to which the trypsin has been inhibited. Examples of this technique can be found in published U.S. Patent Applications 2001/0055816 A1, 2002/0004219 A1, and 2003/0125577 A1. In these published applications, a known amount of trypsin on a substrate capable of producing a detectable response is added to a sample of urine. The substrate is cleaved by trypsin to yield detectable byproducts. If trypsin inhibitors are present, the response is diminished since some of the available substrate is not cleaved. Thus, by measuring the amount of the trypsin present and functioning relative to the amount added, the UTI content can be determined. This method detects any inhibitor of trypsin activity and is non-specific for any given trypsin inhibitor excreted into urine. The patent application published in 2001 discloses the use of a polycarboxylic chelating agent to inhibit the

interference of calcium in the sample. The patent applications published in 2002 and 2003 concern certain aromatic esters of arginine shown to be useful as substrates for trypsin in the method just described.

Another method which determines directly the amount of UTIs in a urine sample involves the development of antibodies which attach themselves to the urinary trypsin inhibitors and which, by the addition of immunoassay reagents containing the antibody to a sample, produce a response signal. There are various immunoassay methods which could be used to apply antibodies of the invention such as microparticle capture immunoassays (MIC), latex agglutination inhibition (LAI), solid phase chromatographic (IC), radioimmunoassays (RIA), enzyme linked immunosorbent assays (ELISA), enzyme linked assays (EIA), fluorescence linked assays (FIA), luminescence linked assays (LIA), rare earth metals label assays, chemiluminescence assays (CLA) and optical color label assays (OA) such as colored latex particle and colloidal gold. It is also feasible to use electrochemical signal transducers (EST) based on amperometric, impedimetric, and potentimetric detection methods.

In principle, immunoassays can be of either a heterogeneous format requiring a separation step or a homogeneous format without separation and either of a competitive or a non-competitive nature. For heterogeneous assays, solid phases can be used to separate bound antigen from free antigen and can include plastic wells, tubes, capillaries, membranes, latex particles, and magnetic particles. Antibodies are attached to the solid phases. Antibodies can also be attached or conjugated (labeled) with reagents that directly or indirectly produce detectable responses, other antibodies or other reagents in a variety of fashions. An immunoassay can also employ multiple and different antibodies in a variety of manners such sandwich assays, double label assays, and multiple sandwich assays depending on the detection needs for the material being detected.

In general, the immunoassay reagents undergo changes whereby a signal is generated and the intensity of the signal generated is proportional to the concentration of the analyte measured in the specimen or sample. Immunoassay reagents contain indicator dyes, metals, enzymes, polymers, antibodies, surface active agents, particles, electrochemically reactive ingredients and various other chemicals dried or filled onto carriers. Carriers often used are tubes, cups, capillaries, strips, vials, papers, microfluidic devices, cassettes, membranes or polymers with various sample volume, uptake and transport properties.

It has been found that polyclonal antibodies produced from rabbits inoculated with urinary trypsin inhibitors purified from the urine of patients with kidney disease are useful in measuring the amount of total UTIs in urine samples from both healthy individuals and those with disease. However, the polyclonal antibodies are not able to distinguish the various forms of UTI from each other, in particular from the pro-inhibitors interleukin-α-inhibitor (I-α-I) and pre-interleukin-α-inhibitor (P-α-I), and the precursor protein for pro-inhibitor biosynthesis (AMBK). Since all these proteins are readily present in the healthy patients, the cross reactivity of the polyclonal antibody makes it useless for distinguishing diseased patients from healthy patients by using blood specimens. In the case of urine specimens, the high molecular weights of P-α-I and I-α-I make it less likely that they will pass through the kidney, thus the polyclonal antibodies were more effective in spite of their reduced specificity. However due to the non-specific nature of the polyclonal antibodies they have not been found to be more effective than the general enzyme inhibition method previously discussed. The polyclonal antibodies were cross-reactive to all forms of UTI in urine and not specific to any one form.

In Journal of Immunoassay 1991; 12:347-69, Trefz et al disclose the use of monoclonal antibodies in an enzyme-linked immunoassay (ELISA). The results showed that the method was effective to distinguish between healthy individuals and those with disease, since the level of UTIs in the urine of those individuals with disease was higher than those without disease. The authors noted that Inter-α-trypsin inhibitor (ITI) having a molecular weight of about 240 kDa, contained several lower molecular weight peptides, understood to result from disintegration of the ITI.

During the cross-reactivity studies of the polyclonal antibody for UTI, we were surprised to discover that further breakdown of Bikunin occurs during the acute phase infections in pateints leading to the formation of a UTI containing both Kunitz inhibitor domains, but lacking the chondroitin sulfate chain. This UTI, termed Uristatin, has a molecular weight of ~17 kDa. In a paper (Clinical Chemistry Acta (2003) in press) reporting tests with a dipstick for detecting urinary trypsin inhibitors, Pugia et al showed that the dipstick reported the presence of two forms for UTIs; Bikunins and Uristatins. They identified the typical molecular weight of Bikunin (30.9 kDa) and three key forms of the Uristatin, designated Uristatin-1 (5.9 kDa), Uristatin-2 (8.5 kDa) and the combination of Uristatin1 and Uristatin 2 which was termed Uristatin (17.4 kDa). All forms of Uristatin lack the chondroitin sulfate chain, and are very prevalent in patient specimens when analyzed by electrophoresis. It was also noted that, given the conditions of the determination, molecular weights of UTIs could vary considerably. Additional variations of Bikunin and Uristatin were also noted between patients specimens, which may be due to fragmentation of parts of peptide structure, variations in the peptide structure and variations in the sugar side chains.

There remains a need for a method of measuring specific urinary trypsin inhibitors that is able to detect the presence of disease by determining certain

characteristic UTIs not found in healthy individuals. The present inventors have developed a method for making such determinations by using certain monoclonal antibodies, as will be described in detail below.

Summary of the Invention

The invention includes certain monoclonal antibodies that are able to attach themselves to urinary trypsin inhibitors (UTIs) characteristic of disease in humans and that can be detected by standard analytical techniques, including MIC, LAI, IC, ELISA, EIA, RIA, LIA CLA, OA and EST (acronyms defined above) as both heterogeneous and homogeneous assays. These monoclonal antibodies are characterized by their ability to allow measurement of specific urinary trypsin inhibitors such as Bikunin, AMBK, Uristatin or fragments of Uristatin designated as Uristatin-1 and Uristatin-2 in the presence on pro-inhibitor forms such as I-α-I and P-α-I .

Specific measurement of a UTI may be obtained by either using antibodies that recognize only that particular UTI or by subtraction using antibodies that recognize other UTIs from a measurement that measures all or most of the prevalent UTIs. In the examples below, antibodies secreted by hybridoma ATCC 420-5D11.5G8.1E4, hybridoma ATCC 421-3G5.4C5.3B6 and hybridoma ATCC 421-5G8.1A8.5C1 are shown to be suitable for determining the amount of UTIs in both urine and blood. Additional antibodies with the required properties are expected to be found.

Such monoclonal antibodies may be made by introducing purified UTIs into mice as an immunogen. Single hybridoma clones producing only one antibody are created by carrying out the procedure of Kohler and Milstein, and then the antibodies having the desired characteristics are determined by using the ELISA method or other immunoassay techniques such as those described above.

In another aspect, the invention includes methods of using the novel monoclonal antibodies to detect and measure forms of urinary trypsin inhibitors of interest, that is, those characterizing persons having disease. Where a monoclonal antibody is specific to a UTI, e.g. to Bikunin, Uristatin, or AMBK, the UTI can be measured directly. When a monoclonal antibody is able to bind to more than one UTI, then by using more than one antibody, the content of a particular UTI of interest may be determined by difference.

Analyses may be carried out on samples of many biological fluids, including but not limited to blood, urine, water, saliva, spinal fluid, intestinal fluid, food, and blood plasma. Blood and urine are of particular interest.

Description of the Preferred Embodiments

Definitions

Since the literature has employed various names for enzyme inhibitors, the following will provide definitions for the terms as used herein.

Urinary trypsin inhibitors (UTIs) means all the inhibitors identified as inhibiting the serine proteases, including without limitation such as Bikunin, HI-30, AMBK, Uristatin, Uristatin 1, and Uristatin 2.

HI-30 means a fragment of higher molecular weight urinary trypsin inhibitors that may or may not be identical to bikunin. Its molecular weight is reported to be 30 kDa (range of 25 to 35 kDa).

AMBK means the precursor protein which has three connected proteins namely the inhibitor portion (HI-30) plus alpha-1-microglobulin plus a 25kDA unidentified protein and has a molecular weight of about 63 kDa (range of 40 to 80 kDa) .

Bikunin means a protein consisting of one or two proteinase inhibitor domains of the Kunitz type that can be connected by a short peptide chain and extended by N- and

C- terminated polypeptide chains. The protein is linked to a sulphated chondroitin chain and to an olegosaccharide and has a molecular weight of about 30 kDa (range of 25-35 kDa). The structure is shown by Pugia et al in Clinical Biochemistry 32 (2002) 105-110 and the 2000 paper by Fries and Blom in the International Journal of Biochemistry & Cell Biology discussed above. The calculated molecular weight of Bikunin is 30.9 kDa in the presence of both the chondroitin sulfate chain (13.5 kDa) and the sugar side chains (1.9 kDa); these values are based on previously published sequences for HI-30.

Uristatin means a protein fragment of Bikunin resulting from cleaving the chondroitin sulfate chain from Bikunin and having a molecular weight of about 17 kDa (range of 12 to 23 kDa). The calculated molecular weight of Uristatin is 17.4 kDa lacking the chondroitin sulfate chain (13.5 kDa); these values are based on previously published sequences for HI-30.

Uristatin 1 and 2 mean protein fragments of uristatin which contain either the Kunitz type inhibitor domains 1 or 2 and having a molecular weight of about 6 kDa (range of 3 to 9 kDa) and about 8.5 kDa (range of 5.5 to 11.5 kDa) The calculated molecular weight of Uristatin 1 and 2 are 5.9 kDa and 8.5 kDa respectively; the values are based on previously published sequences for HI-30.

Pro-inhibitors mean the non-inhibitor form of the protein precursor of Urinary trypsin inhibitors, including without limitation such as proteins such as I-α-I and p-α-I.

I-α-I means Inter-α-inhibitor , understood to refer to Bikunin attached to two heavy chains, which are H_1 and H_2 by means of the chondroitin sulfate chain.

P-α-I means Pre-α-inhibitor, understood to refer to Bikunin attached to one heavy chain, which is H_3 and differs from the two heavy chains in I-α-I.

Monoclonal Antibodies

Monoclonal antibodies are distinguished from polyclonal antibodies in being identical and capable of attaching themselves to a single epitope of an antigen. Polyclonal antibodies, in contrast, are not identical and therefore are not able to provide the precise analysis for which monoclonal antibodies are valuable. Monoclonal antibodies may be produced by the method of Kohler and Milstein, Nature 256:495 (1975). An immunogen (antigen) of interest is injected into mice and the B-cell lymphocytes produced in response to the immunogen are harvested after a period of time. The B-cells are combined with myeloma cells obtained from mice and introduced into a medium which permits the B-cells to fuse with the myeloma cells, producing hybridomas. These fused cells (hybridomas) are then placed in separate wells in microtiter plates and grown to produce monoclonal antibodies. The monoclonal antibodies are tested to determine which of them are suitable for detecting the antigen of interest. After being selected, the monoclonal antibodies can be grown in cell cultures or by injecting the hybridomas into mice.

Making and Separating Preferred Antibodies

Mice are injected with purified UTIs as the analytical target. Antibody producing cells are taken from the animals. Antibody-producing cells are fused with cells that grow continuously in culture to form hybridomas. A single hybridoma produces only one antibody. A single hybridoma divides to produce a large population of 'clones' all making the same "Monoclonal" antibody. Living hybridomas are frozen indefinitely in liquid nitrogen.

The particular procedures used by the present inventors to prepare monoclonal antibodies are described in detail in the examples. It will be understood by those skilled

in the art that those procedures can be modified or augmented and that they are not to be considered to limit the scope of the invention. For example, other techniques for both immunization and fusion protocols which yield hybridomas are familiar to workers in the field of monoclonal antibodies.

The present invention involves monoclonal antibody clones which were selected on the basis of their ability to bind to one or more UTIs and also preferentially to bind to certain epitopes on the intact molecule which then appear on particular fragments once the break-down of the uristatin occurs.

Detecting Trypsin Inhibitors in Urine

Once the selective monoclonal antibodies have been located, they may be grown by conventional procedures and used in ELISA testing, or any other immunoassay technique described earlier to detect the presence of the UTI that are characteristic of infection and/or inflammation.

ELISA (enzyme-linked immunosorbent assay) provides a very sensitive method for detecting antigens. In an antigen down ELISA, a microtiter plate receives a sample suspected of containing a certain antigen. After allowing for adsorption of the uristatin onto the plate, and washing all non-bound materials off, the monoclonal antibody is added, incubated so that it can bind to the uristatin and the excess washed off. The monoclonal antibody added may have already been labeled with a reporter molecule to permit the generation of a signal to be read by any number of techniques. Alternatively a ligand capable of attaching to the antibody (e.g.an anti-mouse antibody conjugated to a reporter molecule) is added. After excess of the enzyme-coupled ligand has been washed off, the chromogen or other substrate is added if necessary and the color developed used as an indicator of the amount of antigen present.

There are various immunoassay methods that could be used to apply antibodies of the invention such as microparticle capture immunoassays (MIC), latex agglutination inhibition (LAI), solid phase chromatographic (IC), radioimmunoassays (RIA), enzyme linked immunosorbent assays (ELISA), enzyme linked assays (EIA), fluorescence linked assays (FIA), luminescence linked assays (LIA), rare earth metals label assays, chemiluminescence assays (CLA) and optical color label assays (OA) such as colored latex particle and colloidal gold. It is also feasible to use electrochemical signal transducers (EST) based on amperometric, impedimetric, and potentimetric detection methods.

Example 1
Preparation of Monoclonal Antibodies

BALB/c mice were immunized with 100 µg/mouse of a uristatin peptide obtained from SciPac Ltd. Sittingbourne, Kent, UK, product code P250-1 to produce a response. After one month, ocular bleeds were taken from each mouse and titered by ELISA against the uristatin peptide to assess the immune response. The mice showing the best response were boosted by injection of 100 µg/mouse with the uristatin peptide. After four days, mice were sacrificed and their spleens used for fusion according to the method of Kohler and Milstein, Nature 256:495 (1975). The spleenocytes were fused with SP2-0 Ag14 myeloma cells using PEG (polyethylene glycol) solution with a ratio of spleenocytes to Myeloma cells of 5:1 and plated into 96 well plates using 50% PEG/HAT growth media. After 7-10 days of incubation at 37 degrees Celsius, fusion cultures were monitored for growth by feeding every 3-4 days utilizing the HAT (hypoxanthine, aminopterin, thymidine) selection method followed by subculturing with HAT growth media.

After 2-3 weeks, the wells having hybridoma colony growth were tested by ELISA to determine which growths produced an antibody immune response to the uristatin peptide. The 96 well plate cultures were tested with Uristatin (17.4 kDa) at 1ug/mL coated plates. After coating plates overnight at 2-8°C, all plates were washed and blocked. Cell culture supernatants were then applied 100µl/well for one hour at room temperature. After washing plates, Goat anti-mouse IgG Horse Radish Peroxidase at 1:2000 dilution was applied at 100uL/well for one hour. Plates were washed once again followed by OPD (o-phenylene diamine dihydrochloride) substrate and read at 490nm on a Spectra Max plate reader.

The colonies giving a positive response were transferred to 24 well plates for further expansion and retesting to verify the positive results. The colonies testing positive were further expanded in six well plates in Iscove's Modified Dulbecco's Medium (IMDM) with 10% Fetal Bovine Serum (FBS). After expansion, the colonies were frozen at - 70°C and then transferred to liquid nitrogen for long-term storage.

Based on ELISA results using the Uristatin various clones were further expanded in IMDM, 10% FBS and frozen down.

Example 2
Screening Procedure of Polyclonal Antibodies

Rabbit polyclonal antibodies raised against purified Uristatin were used in the screening process as this antibody was expected to be non-specific for any given form of Uristatin. Serum and urine specimens from three patients with infection and two healthy controls were characterized by Western Blot tests using these polyclonal antibodies. The Western blot tests used the commercial pre-cast gel system (Invitrogen, San Diego CA). Urine and plasma specimens were loaded with 1 µg and 5 µL per lane. The western blots were stained with a WesternBreeze® chromogenic immunodetection kit (from

Invitrogen) following the manufacturer's instructions. Rabbit anti-uristatin antiserum was used at a dilution of 1:250 000 as the primary antibody in the western blot analysis (See Table 1). These Western Blot results demonstrated that the rabbit polyclonal antiserum body detected Uristatin, Bikunin, P-α-I, I-α-I, and AMBK.

Table 1. Results of Western Blot analysis using polyclonal serum as interogating antibody of specimens

Specimen	Major proteins observed in urine	Minor proteins observed in urine	Major proteins observed in plasma	Minor proteins observed in plasma
Patient 1	Uristatin	Bikunin, AMBK	P-α-I, I-α-I,	AMBK, Bikunin
Patient 2	Bikunin	Uristatin, AMBK	P-α-I,	AMBK, Bikunin
Patient 3	Bikunin	Uristatin, AMBK	P-α-I,	I-α-I, AMBK, Bikunin
Healthily control 1	None	None	P-α-I,	I-α-I, AMBK
Healthily control 2	Bikunin	None	P-α-I, I-α-I,	AMBK, Bikunin

These Western Blot tests also demonstrated that Uristatin was better able to differentiate diseased patients from health controls than Bikunin, P-α-I, I-α-I and AMBK. The results showed that Bikunin was found in the urine of the three patients with infections but also in the urine of one control subject. We found Uristatin in all three patients but not any of the control subjects. Also we found marked increase of Uristatin in one patient. This supports our belief that specific forms of UTIs can be more related to disease in certain patients than others. It should be noted here that Uristatin or Bikunin could not be measured in blood because their concentration is much lower than the pro-inhibitors I-α-I and P-α-I. The pro-inhibitors do not pass through the kidneys into urine in significant amounts, making it easier to detect Uristatin and Bikunin in urine than in blood. The high cross reactivity of the polyclonal method does not allow detection of the lower concentration of Uristatin in blood.

The presence of urinary trypsin inhibitors has been shown to be meaningful in a number of diseases. These disease include infection (pneumonia, urinary tract infection,

upper respiratory tract infection, neonatal sepsis, meningitis, appendicitis, bacterial infections, bone and joint infections), acute inflammation (trauma, kidney stones, surgical truama, cardiopulmonary bypass, myocardial infarction, and burns), chronic inflammation (rheumatoid arthritis, lung emphysema, inflammatory bowel disease, pancreatitis, Crohn's disease, and as a risk factor for cardiovascular disease), neoplasia (leukemia, multiple myleoma, lymphoma (non-hodgkins), ovarian cancer, breast cancer, pancreatic cancer, stomach cancer, colon cancer), and kidney disease.

Clinical value is assessed by the ability of a method to detect the urinary trypsin inhibitor forms more prevalent in disease. Also methods able to work not only in urine but also in blood would be of additional clinical value. Blood is the preferred fluid for clinical laboratory analysis allowing consistency in results, while urine is the preferred fluid for allowing non-invasive sampling at the point of care.

Therefore an immunoassay which detects only specific forms of UTI's, such as Uristatin or Bikunin and not P-α-I or I-α-I would have clinical utility. The results of our tests also showed that no detectable P-α-I or I-α-I was in urine but was present in all plasma specimens from controls and patients. Any immunoassay of blood for active UTIs such as Uristatin or Bikunin should not detect P-α-I or I-α-I. The predominant bands in blood corresponded approximately to an AMBK of 63 kDa, P- α-I of 115 to 125 kDa, and I-α-I of 220 kDa in both patients and controls.

All the Uristatin monoclonal antibody clones were screened based on their ability to bind Uristatin in the ELISA assay using the procedure described in Example 1 and the patterns observed in Western Blot tests for antibodies using patient and healthy specimens as described in Table 1. The antibodies were grouped by similarity of patterns into three groups and the best representative antibodies of each group selected based on ELISA testing against the uristatin standard. The selection of clones for further

study were as follows, from group A: 421 - 3G5 from group B: 421 - 5G8 and from group C: 420 – 5D11. For the selected mother colonies, cell culture supernatants were cloned out twice using the limiting dilution method assure that the antibodies were monoclonal. The hybridomas secreting these monoclonal antibodies in Iscove's Modified Dulbecco's Medium (IMDM) 30% Fetal Bovine Serum (FBS), and 10% Dimethyl Sulfoxide (DMSO)have been deposited at the American Type Culture Collection and designated as ATCC 420-5D11.5G8.1E4, ATCC 421-3G5.4C5.3B6, and ATCC 420-5G8.1A8.5C1.

Example 3
Characterization of Monoclonal Antibodies Against Purified Uristatin and Bikunin Standards

The urinary trypsin inhibitors were produced by SciPac Ltd for our study (Sittingbourne, Kent, UK, product code P205-1); these are purified from the urine of patients with chronic renal failure. The standards were characterized by SDS-PAGE. We used a commercial pre-cast gel system (Invitrogen, San Diego CA) 4-12% NuPAGE® Bis-Tris with a MES (2-morpholinoethanesulfonic acid) running buffers (reducing and non-reducing), following exactly the manufacturer's procedure. Specimens were loaded at 2 μg per lane. Estimations of the proteins' molecular weights were based on a full set of standards: MagicMark12™ (Invitrogen) and SeeBlue®Plus2 (Invitrogen). Protein bands were stained with Colloidal Blue® (Invitrogen).

UTI lot 20-120 contained mostly 30.9 kDa material (~80%) with a significant amount of AMBK having a molecular weight of ~63 kDa. UTI lot 124-111 had 80% of the material as the 17.4 kDa band (Uristatin) but also contained amounts of Uristatin-1 (5.9 kDa) and a significant amount of Bikunin (30.9 kDa) but no AMBK was seen to be present. UTI lot 80-117 containing substantially only the 17 kDa Uristatin material.

The urine samples for the controls were assayed by the ELISA assay described in Example 1 using the three purified monoclonal antibodies and a Goat Anti-Mouse secondary antibody conjugate. The results for group A clone 421 – 3G5, group B clone 421 - 5g8 and group C clone 420 – 5d11 are shown in Table 2 and compared to the polyclonal antibody as a control.

The measurements made of color developed by the OPD substrate indicated the amount of the monoclonal antibody bound to UTIs in the standards just described, whose contents were determined by molecular weights using the Western Blot method. The values were normalized using the polyclonal antibody values and are presented in the table below as percent relative to the polyclonal antibody results.

Table 2. ELISA Results with Specimens from Patient and Healthy Individual and Protein Standards for UTIs.

Specimen	ANTIBODY GROUP B CLONE 421 - 5G8	ANTIBODY GROUP C CLONE 420 – 5D11	ANTIBODY GROUP A CLONE 421 – 3G5	POLYCLONAL ANTIBODY
UTI Standard lot 20-120	92%	100%	71%	100%
UTI Standard lot 124-111	100%	13%	100%	94%
UTI Standard lot 80-117	5%	0%	48%	74%

The monoclonal antibody 421-5G8 bound strongly to UTI lots #124-111 and #20-120 to a similar degree, but only bound weakly to Lot #80-117. This would be consistent with binding to the 35-45 kDa material (Bikunin) in both lots but not in lot 80-117 containing only the 17 kDa material (Uristatin). Thus, this antibody appears specific for Bikunin over Uristatin. While not wanting to be limited to a mechanism, it is believed that binding by this monoclonal antibody could occur through the sulfated chondroitin chain as these moieties are known to possess high antigen affinities for antibodies.

The monoclonal antibody 420-5D11 bound strongly to UTI lot #20-120, very weakly to lot #124-111, and did not bind to lot 80-117. This is consistent with binding to only the 60-80 kDa material (AMBK) in lot #20-120, since only lot #20-120 contains a large amount of this material. If the antibody were specific for Bikunin also, one would expect it to be bound strongly to lot #124-111, which contained about 20% Bikunin. This antibody appears specific for AMBK over Bikunin and Uristatin.

The monoclonal antibody 421 – 3G5 bound strongly to all UTI lots and similar to the non-specific polyclonal antibody. Thus 421-365 would also be a measure of total UTI.

Example 4
Testing of Monoclonal Antibodies with Clinical Specimens

Urine samples were obtained from patients having bacterial infections and from control patients having no such infections. We collected urine specimens from patients without infections; these are the "group 1" patients. To be included in the study, we only required a negative urine and blood culture (10^5 organisms/mL) and a normal blood white blood count (CBC). The second group of patients ("group 2") included those with infections either upper respiratory tract or urinary tract, a conclusion based on a positive complete blood count (CBC) in all. Clean-catch midstream urine collections were obtained from all patients and controls. Specimens were stored at 4°C until tested; but if not tested within 24h, storage was at –70 °C until tested. For all subjects, the evaluation of urine sediment, gram stain and urine microbiological culture were always performed on the day of collection. We collected EDTA-anticoagulated blood from group 2 and performed a CBC, a high sensitivity CRP (Dade Behring, Immunoassay for C-reactive protein) test, and a blood culture; we performed all these tests on the day of blood collection.

The urine samples were also assayed by an ELISA assay using the same polyclonal antibodies as in Example 2. The antibodies were immobilized in polystyrene membrane wells of the high binding microtiter plates (PN 3690 Corning Life Sciences, Acton, MA), then wells were coated with Super Block (Pierce Chem Co., Rockford IL) to ensure none of the following additions attach directly to the plate, then contacted with a urine sample from each patient to bind the antibodies to the Uristatin in the sample. The Uristatin-antibody complex was reacted with a second antibody (goat anti rabbit antibody) conjugated to alkaline phosphatase, then the unbound conjugated antibody was washed away with TBS/2 % TWEEN-20 (ELISA Wash buffer, Upstate Cell Signaling Solutions, Lake Placid NY) and the immobilized antibody assayed by determining the alkaline phosphatase on the plate by hydrolysis of PNPP (*p*-Nitrophenyl Phosphate, Disodium Salt) forming an absorbance at 405 nm (yellow) color upon addition of Diethanolamine Buffer (Pierce Chem Co.). The absorbance is measured a spectrophotometric micro titer-plate reader (SpectroMax. Molecular Devices Corporation, Sunnyvale, CA).

It was found that three control urine samples contained 51.4, 27.9 and 24.8 μg/mL of UTI proteins (Bikunin, Uristatin, and AMBK), while two samples from patients having bacterial infections contained 148 and 49.5 μg/mL. This result confirms the previous Western Blot test findings that polyclonal antibody gave poor separation of patients with infections from healthy controls as would be expected by the cross-reactivity to proteins (Bikunin, Uristatin, and AMBK).

Measurements of UTI's in the urine and plasma of patients can be made specific by the direct method and the indirect subtraction method using monoclonal antibodies, such as those shown in Table 2. As an example of direct method, Bikunin in either a urine or blood specimen can be measured directly with clone 421 - 5G8 over the other

UTI and pro-inhibitors present. An additional example of direct method, AMBK in either a urine or blood specimen can be measured directly with clone 420 – 5D11. An example of indirect method would be subtraction of one or more specific results from a total measurement of UTI. For example, to measure the Uristatin in patient 1's urine, Bikunin would be measured directly with clone 421 - 5G8 and AMBK measured directly with clone 420 – 5D11 and both would be subtracted from a total UTI to arrive at the amount uristatin. Total measurement could be made by the polyclonal antibody as discussed above, monoclonal antibody clone 421 – 3G5 or the enzyme inhibition method. In the case of the polyclonal antibody, it was shown that this total was affected by the pro-inhibitor amounts, therefore the monoclonal antibody clone 21 – 3G5 or enzyme inhibition method would be preferred.

The monoclonal antibodies used in these examples and the conclusion are arrived at are based on cross-reactivity testing of the UTI samples shown in Table 2. It is expected that additional testing will provide more specific information about the UTIs and their response to these in other monoclonal antibodies. The methodology, however, to determine the specific UTIs is not expected to change. That is, certain monoclonal antibodies will be specific to individual UTIs, others to more than one UTI. By using more than one monoclonal antibody, and/or polyclonal antibodies or other methods for determining all UTIs present, a specific UTI can be identified by difference.

WHAT IS CLAIMED IS:

1. A monoclonal antibody for detecting urinary trypsin inhibitors in the urine of persons having disease, said antibody comprising an antibody combining site which binds substantially only to at least one member of the group of urinary trypsin inhibitors (UTI) consisting of AMBK, Bikunin, Uristatin, Uristatin-1, and Uristatin-2.

2. A monoclonal antibody of Claim 1 wherein said UTI is Bikunin.

3. A monoclonal antibody of Claim 1 wherein said monoclonal antibody is secreted by hybridoma ATCC 421-5G8.1A8.5C1.

4. A monoclonal antibody of Claim 1 wherein said UTI is AMBK.

5. A monoclonal antibody of Claim 4 wherein said monoclonal antibody is secreted by hybridoma ATCC 420-5D11.5G8.1E4.

6. A monoclonal antibody of Claim 1 wherein said UTI is Uristatin.

7. A monoclonal antibody of Claim 1 wherein said UTI is Uristatin-1.

8. A monoclonal antibody of Claim 1 wherein said UTI is Uristatin-2.

9. A monoclonal antibody for detecting urinary trypsin inhibitors in the urine of persons having disease, said antibody comprising an antibody combining site which binds to AMBK, Bikunin, and Uristatin.

10. A monoclonal antibody of Claim 9 wherein said monoclonal antibody is secreted by hybrioma ATCC 421-3G5.4C5.3B6.

11. A method of assaying human urine for urinary trypsin inhibitors comprising contacting a sample of said urine with at least one monoclonal antibody capable of binding to urinary trypsin inhibitors characterizing the urine of persons having disease.

12. A method of Claim 11 wherein said urinary trypsin inhibitors characterizing the urine of persons having disease are members of the group consisting of AMBK, Bikunin, Uristatin, Uristatin-1, and Uristatin-2.

13. A method of Claim 12 wherein said monoclonal antibody binds substantially only to AMBK.

14. A method of Claim 12 wherein said monoclonal antibody binds substantially only to Bikunin.

15. A method of Claim 12 wherein said monoclonal antibody binds to AMBK, Bikunin and Uristatin.

16. A method of Claim 12 wherein Bikunin is bound to a first monoclonal antibody capable of binding substantially only to Bikunin, AMBK is bound to a second monoclonal antibody capable of binding substantially only to AMBK, the total content of said AMBK, Bikunin, and Uristatin is bound to a third monoclonal antibody or a polyclonal antibody, or measured by enzyme inhibition, and Uristatin is determined by the difference in the amounts of said bound first and second antibodies and the total content of AMBK, Bikunin, and Uristatin.

17. A method of Claim 13 wherein said monoclonal antibody is secreted by hybridoma ATCC 420-5D11.5G8.1E4.

18. A method of Claim 14 wherein said monoclonal antibody is secreted by hybridoma ATCC 421-5G8.1A8.5C1.

19. A method of Claim 15 wherein said monoclonal antibody is secreted by hybridoma ATCC 421-3G5.4C5.3B6.

20. A method of assaying human urine for urinary trypsin inhibitors comprising the steps of:

(a) adding a urine sample suspected of containing urinary trypsin inhibitors to a substrate;

(b) adding to said sample of (a) monoclonal antibodies capable of binding to urinary trypsin inhibitors characterizing the urine of humans having disease;

(c) adding to the combined monoclonal antibodies of (b) and the urine sample of (a) a ligand capable of binding to said monoclonal antibodies, said ligands being bound to an enzyme;

(d) washing from the combined monoclonal antibodies of (b), the urine sample of (a) and the ligand of (c) the portion of said ligand unbound to said monoclonal antibodies;

(e) determining the amount of said urinary trypsin inhibitors bound to said monoclonal antibodies and said ligands by adding a reporter molecule capable of developing a signal by reaction with said enzyme and correlating the signal developed with the amount of said urinary trypsin inhibitors.

21. A method of Claim 20 wherein said urinary trypsin inhibitors characterizing the urine of persons having disease comprise at least one member of the group consisting of AMBK, Bikunin, Uristatin, Uristatin-1, and Uristatin-2.

22. A method of Claim 21 wherein said monoclonal antibody binds substantially only to AMBK.

23. A method of Claim 21 wherein said monoclonal antibody binds substantially only to Bikunin.

24. A method of Claim 21 wherein said monoclonal antibody binds substantially only to AMBK, Bikunin, and Uristatin.

25. A method for Claim 21 wherein Bikunin is bound to a first monoclonal antibody capable of binding substantially only to Bikunin, AMBK is bound to a second monoclonal antibody capable of binding substantially only to AMBK, the total content of said AMBK, Bikunin, and Uristatin is bound to a third monoclonal antibody or a polyclonal antibody, or measured by enzyme inhibition, and Uristatin is determined by

the difference in the amounts of said bound first and second antibodies and the total content of AMBK, Bikunin, and Uristatin.

26. A method of Claim 22 wherein said monoclonal antibody is secreted by hybridoma ATCC 420-5D11.5G8.1E4.

27. A method of Claim 23 wherein said monoclonal antibody is secreted by hybridoma ATCC 420-5G8.1A8.5C1.

28. A method of Claim 24 wherein said monoclonal antibody is secreted by hybridoma ATCC 421-3G5.4C5.3B6.

29. A method of assaying human urine for urinary trypsin inhibitors characterizing the urine of humans having disease comprising the steps of:

(a) adding a sample of urine to sulfate-polyacrylamine gel and carrying out electrophoresis to separate the proteins in said sample;

(b) transferring the proteins to a nitro-cellulose membrane using electrophoretic blotting.

(c) adding monoclonal antibodies capable of binding to urinary trypsin inhibitors found in the urine of humans having disease, said monoclonal antibodies being characterized by their ability bind to urinary trypsin inhibitors characterizing the urine of humans having disease;

(d) determining the characteristic molecular weight bands in said separated proteins;

(e) identifying said urinary trypsin inhibitors characterizing the urine of humans having disease.

30. A method of Claim 29 wherein said urinary trypsin inhibitors characterizing the urine of persons having disease comprise at least one member of the group consisting of AMBK, Bikunin, Uristatin, Uristatin-1, and Uristatin-2.

31. A method of Claim 30 wherein said monoclonal antibody binds substantially only to AMBK.

32. A method of Claim 30 wherein said monoclonal antibody binds substantially only to Bikunin.

33. A method of Claim 30 wherein said monoclonal antibody binds substantially only to AMBK, Bikunin, and Uristatin.

34. A method of Claim 30 wherein Bikunin is bound to a first monoclonal antibody capable of binding substantially only to Bikunin. AMBK is bound to a second monoclonal antibody capable of binding substantially only to AMBK. The total content of said AMBK, Bikunin, and Uristatin is bound to a third monoclonal antibody or a polyclonal antibody, or measured by enzyme inhibiting and Uristatin is determined by the difference in the amounts of said bound first and second antibodies and the total content of AMBK, Bikunin, and Uristatin..

35. A method of Claim 31 wherein said monoclonal antibody is secreted by hybridoma ATCC 420-5D11.5G8.1E4.

36. A method of Claim 32 wherein said monoclonal antibody is secreted by hybridoma ATCC 420-5G8.1A8.5C1.

37. A method of Claim 33 wherein said monoclonal antibody is secreted by hybridoma ATCC 421-3G5.4C5.3B6.

Abstract

Certain monoclonal antibodies are able to detect urinary trypsin inhibitors (UTIs) that are characteristic of disease in humans. In particular, the UTIs include AMBK, Bikunin, Uristatin, Uristatin-1, and Uristatin-2, as defined herein.

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